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Fluorescent Nanodiamonds as Free Radical Sensors in Aging Yeast Cells

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General Introduction

Kiran J. van der Laan

Diamonds last forever, humans do not. In order to investigate the decay of a human in the so-called process of aging, we propose fluorescent nanodiamonds as a biosensor to unravel the role of free radicals in this process. In this introduction I will guide you through the different fields that are involved in this highly interdisciplinary research.

Aging and free radical formation

Aging is defined as a time-dependent functional decline, which is actually the major risk factor for disease and death after age 28 in the developed countries.[1,2] In the current population, there are more and more people that are living longer; the so-called double aging phenomenon. Therefore, there is not only a scientific relevance for unraveling the process of aging on a molecular level, but as well a societal relevance due to the so-called double aging process (more people are living longer).

Figure 1. The Hallmarks of Aging. This schematic gives an overview of the nine mechanisms known to be involved in aging (reprint from López-Otín et al. 2013[1]).

Although some causes of aging have been proposed (figure 1), there is surprisingly little known on a molecular level. One of the theories aiming to

explain cellular aging, is the traditional free radical theory of aging. This theory states that the accumulation of free radicals, as a result of mitochondrial dysfunction, is responsible for the cellular damage that occurs with aging.[2] In the last decade, conflicting evidences have shown that this process is not that one-sided and that ROS can have both positive and negative effects on cellular health. The current theory acknowledges that there is a basal level of ROS, which is needed in normal metabolism where they function as signaling molecules. Whenever this balance is disturbed to a certain threshold, the unbalanced ROS levels start to initiate negative effects which can result in all kinds of diseases and also contributes to cellular aging.

Free radicals are molecules with a free electron in their outer orbit, which makes them highly reactive and short-lived. Therefore they are very challenging to detect, which makes it difficult to gain information on free radical formation and spreading. This expresses the need for a method to determine the location of free radicals.

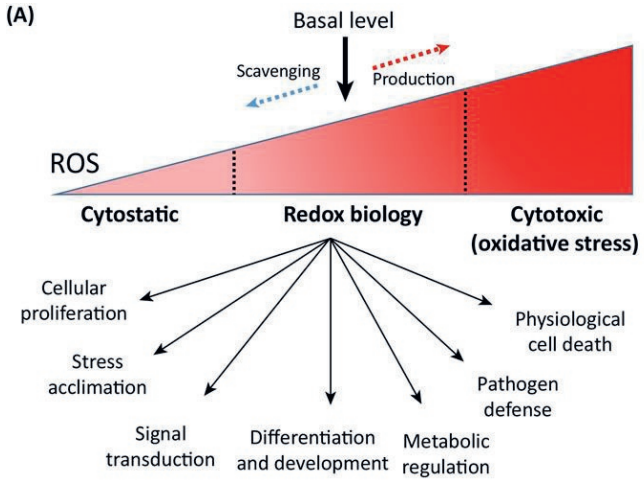

Figure 2. Balancing ROS generation and ROS scavenging (reprint from Mittler 2017[3]).

Free radical detection by diamond magnetometry

Using a new method called diamond magnetometry, we aim to monitor free radical activity using fluorescent nanodiamonds (FNDs). The FNDs are not only fluorescent, but their fluorescence also responds to external factors in the

surrounding (e.g. magnetic signals) by showing fluctuations in the fluorescent signal. Using the magnetic properties of FNDs, a magnetic resonance signal is converted into an optically detectable signal. As explained, it is currently still challenging to find methods to determine the exact location and mode of action of free radicals. In diamond magnetometry, the advantages of both fluorescence imaging and magnetic resonance techniques are combined, while eliminating some of the disadvantages. Like in magnetic resonance, diamond magnetometry can be non-destructive and element- (or radical-) specific. Because of the fluorescence, diamond magnetometry is sensitive (up to single photon detection[4]) with high spatial resolution, without the need of complex and extensive equipment as with MRI. Moreover, diamonds are forever and do not suffer from photo-bleaching, allowing for long-term live cell imaging experiments. So we have the best of both worlds.

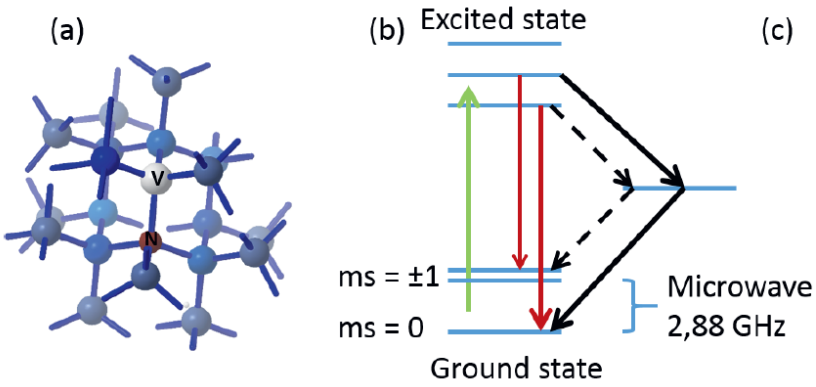

Figure 3 Fluorescent Nanodiamond. A: Diamond lattice with NV center. B: Energy diagram of NV center.[5]

The nanodiamonds used here are diamonds of nanosize, mostly 70 nm in this study, that contain so-called NV centers: two carbon atoms are replaced by a nitrogen atom (N) and a vacancy (V) next to it (Figure 3). These NV centers have a spin state that can be read out optically, since their fluorescence depends on the surrounding magnetic field. After excitation with a green laser, the NV center emits red photons. Coming from the ms=±1 spin state there is an alternative route to the ground state over a dark state. This alternative route over the dark state is observed by a decrease in fluorescence (due to fewer red photons that are emitted). Free radicals have free electron spins and thus

produce a magnetic field or spin flipping which influences the population of these states. This means that a magnetic signal in the surrounding of the diamond (e.g. coming from free radicals) is converted into an optical signal, and can be read out by optical detection of the fluorescence. Hence, there is no need for expensive and complicated MRI machines. [6–8]

Using a home-built, customized confocal microscope, detection and interpretation of the signals can be done in multiple ways. Two of them are explored here: Optically Detected Magnetic Resonance (ODMR) measurements, which involve applying microwaves at the resonance frequency, or by t1 relaxation measurements, which include applying a laser pulse to polarize the orientation of the diamonds and subsequently measuring the required time until relaxation.

Yeast as a model system for aging research

In this research we used *Saccharomyces cerevisiae* **(**or baker's yeast) as a model system. Baker's yeast is an important model organism to study a wide range of biological processes. In particular, they are one of the favored model organisms to study aging for numerous reasons.

First of all, an important part of the aging process on a molecular level has been highly conserved among different organisms (from yeast to multicellular eukaryotes, including humans). Additionally, they are convenient to work with, easy and fast to culture, and they are highly suitable for genetic manipulations.[9] Another useful characteristic, is the fact that you can model different effects of aging in yeast.[10] They can age by both replicative and non-replicative processes (Figure 4). The former are relevant to mimic aging of quickly dividing cells, which undergo cell death after a certain (fixed) amount of divisions (such as adult stem cells). This thesis however is focused on the latter, so-called chronological aging, which can be used to mimic aging in cells that do not divide any more (e.g. nerve cells). Furthermore, due to asymmetric cell division, it is relatively easy in yeast to differentiate and separate young and old cells due to their size difference.[11,12]

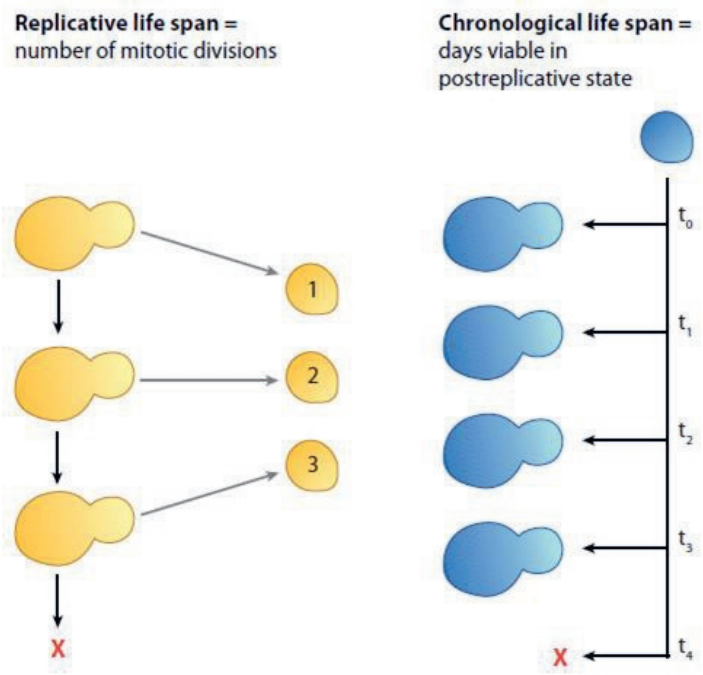

Figure 4. Schematic representation of replicative and chronological aging in yeast cells. RLS= the cumulative number of mitotic divisions a cell can undergo. CLS= the amount of time a cell can remain viable while in a non-dividing state (time point at which cells are unable to reenter the cell cycle). (Reprint from Steinkraus et al. 2008.[10])

Thesis objectives and outline

In this thesis, important and necessary milestones are reached on the route towards the application of FNDs as free radical sensors in aging yeast cells. The first step was to get the diamonds inside yeast cells. This is needed to get the diamonds as close to the target (free radicals) as possible, to enable sensing their magnetic fields. Compared to mammalian cells this is a challenge, since yeast cells have a thick cell wall (in addition to the membrane). The method to obtain this internalization is described in **chapter 2**. In order to eventually take actual free radical measurements in aging cells, we evaluated whether the presence of diamonds or the diamond uptake protocol affected the capability of the cells to age. This, as well as the subcellular localization of diamonds after uptake, is described in **chapter 3**. The next research chapter goes into more depth to what happens to the cells after diamond ingestion. We observed that the cells survive

and in **chapter 4** we looked more in detail into the metabolic response of yeast cells to diamond internalization. We investigated if the diamond itself causes oxidative stress, which is important to know to be able to discriminate age-related free radical signals from FND-related free radical signals. As mentioned before, free radicals are involved in many different pathogenic processes. Therefore, next to aging research, there is a broader interest of using FNDs as free radical biosensors in biomedical research (e.g. in cancer research). Therefore, the possibilities and consequences of fluorescent nanodiamonds have been tested in a variety of cell types (**chapter 5**) and in different model organisms (**chapter 6**). Lastly, **chapter 7** discusses the importance and relevance of the data in this thesis, as well as future perspectives and the valorization opportunities of this research in different fields.

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